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The Effect of Cotinine or Cigarette Smoke Co-Administration on the Formation of O^6-Methylguanine Adducts in the Lung and Liver of A/J Mice Treated with 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)

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4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), a tobacco-specific nitrosamine, induces lung adenomas in A/J mice, following a single intraperitoneal (ip) injection. However, inhalation of tobacco smoke has not induced or promoted tumors in these mice. NNK-induced lung tumorigenesis is thought to involve O^6-methylguanine (O^6MeG) formation, leading to GC→AT transitional mispairing and an activation of the K-ras proto-oncogene in the A/J mouse. NNK can be metabolized by several different cytochromes P_{450}, resulting in a number of metabolites. Formation of the promutagenic DNA adduct O^6MeG is believed to require metabolic activation of NNK by cytochrome P_{450}-mediated α-hydroxylation of the methylene group adjacent to the N-nitroso nitrogen to yield the unstable intermediate, methanediazohydroxide. Nicotine, cotinine (the major metabolite of nicotine), and aqueous cigarette tar extract (ACTE) have all been shown to effectively inhibit metabolic activation of NNK to its mutagenic form, most likely due to competitive inhibition of the cytochrome P_{450} enzymes involved in α-hydroxylation of NNK. The objective of the current study was to monitor the effects of cotinine and cigarette smoke (CS) on the formation of O^6MeG in target tissues of mice during the acute phase of NNK treatment. To test the effect of cotinine, mature female A/J mice received a single intraperitoneal injection of NNK (0, 2.5, 5, 7.5, or 10 μmole/mouse) with cotinine administered at a total dose of 50 μmole/mouse in 3 separate ip injections, administered 30 min before, immediately after, and 30 min after NNK treatment. To test the effect of whole smoke exposure on NNK-related O^6MeG formation, mice were exposed to smoke generated from Kentucky 1R4F reference cigarettes at 0, 0.4, 0.6, or 0.8 mg wet total particulate matter/liter (WTPM/L) for 2 h, with a single ip injection of NNK (0, 3.75, or 7.5 μmole/mouse) midway through the exposure. Cigarette smoke alone failed to yield detectable levels of O^6MeG. The number of O^6MeG adducts following ip injection of NNK was significantly ($p < 0.05$) reduced in both lung and liver by cotinine and by cigarette smoke exposure. Our results demonstrate that NNK-induced O^6MeG DNA adducts in A/J mice are significantly reduced when NNK is administered together with either cotinine, the major metabolite of nicotine, or the parental complex mixture, cigarette smoke.

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4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), a tobacco-specific nitrosamine, induces lung adenomas in A/J mice within months after a single intraperitoneal (ip) injection (Hecht *et al.*, 1989). The initial event in NNK-induced lung tumorigenesis is the formation of O^6-methylguanine (O^6MeG), a major promutagenic adduct which leads to GC→AT transitional mispairing and an activation of the K-ras proto-oncogene in the A/J mouse lung (Peterson and Hecht, 1991; Ronai *et al.*, 1993). Formation of O^6MeG from NNK is believed to result from α-hydroxylation of the methlylene group adjacent to the N-nitroso nitrogen by cytochrome P_{450} enzymes (Hoffmann *et al.*, 1994) (Fig. 1).

Although ip injections of NNK (2.5 to 10 μmole/mouse) have been shown to induce lung adenomas in A/J mice (Hecht *et al.*, 1989), inhalation of cigarette smoke has not induced or promoted tumors in this mouse model (Finch *et al.*, 1996). Numerous studies have demonstrated that nicotine can inhibit the metabolic activation of NNK both *in vitro* (Murphy and Heiblum, 1990; Schuller *et al.*, 1991) and *in vivo* (Richter and Tricker, 1994). In a study using snuff, snuff extract, and tobacco-specific nitrosamines (TSNA) to evaluate the induction of oral-cavity tumors in rats, the authors hypothesized the possibility of nicotine acting as a competitive inhibitor of the metabolic activation of the TSNA in snuff (Hecht *et al.*, 1986). Moreover, studies from our laboratory have shown that nicotine, its major metabolite cotinine, and aqueous solutions of cigarette tar all effectively reduce the genotoxicity of NNK in both Ames mutagenicity and sister chromatid exchange assays (Lee *et al.*, 1996). Cigarette smoke is a complex mixture containing thousands of components, some of which are recognized as either substrates or competitive inhibitors of the cytochrome P_{450} enzymes (Murphy and Heiblum, 1990). The evidence suggests that competitive inhibition of cytochromes P_{450} by nicotine, cotinine and/or other tobacco constituents is the most likely mechanism for their observed modulation of the

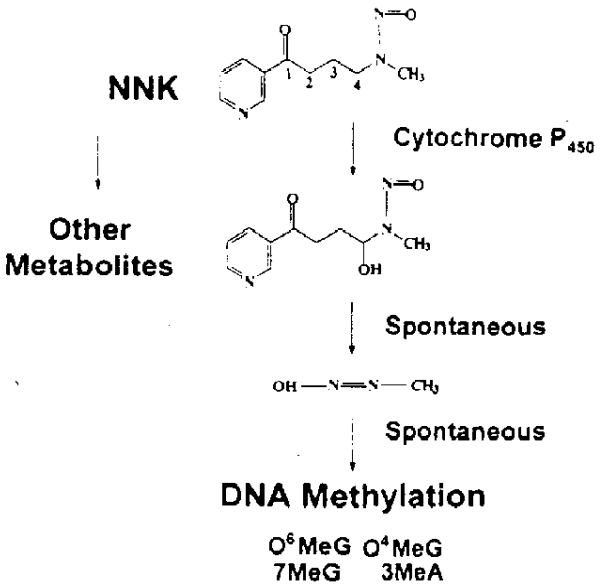

FIG. 1. Metabolic activation of NNK and related DNA adduct formation. NNK is a procarcinogen in laboratory animals and the activating P_{450} enzymes reside mainly in liver and lung. The initial and key step for NNK activation relies on a P_{450}-mediated α-hydroxylation reaction to the unstable metabolite—methyldiazohydroxide—which donates the methyl group to DNA, forming O°MeG. O°MeG is recognized as the promutagenic biomarker for NNK-induced tumorigenesis.

biological effects of NNK (Lee *et al.*, 1996; Murphy and Heiblum, 1990).

High-performance liquid chromatography (HPLC), with a strong cation-exchange column coupled with absorbance and fluorescence detection, is the method of choice for the quantification of methylated DNA adducts produced by NNK (Bedell *et al.*, 1982; Hecht *et al.*, 1986b; Herron and Shank, 1979). We previously optimized sample preparation and detection conditions in our HPLC systems for NNK-induced, methylated adducts (Chang *et al.*, 1996, 1998), and we have applied those optimized methods. Concentrations of lung and liver O°MeG adducts increase rapidly during the first 2 h after the ip administration of NNK, plateau at 4 h and remain relatively stable for at least 24 h (Chang *et al.*, 1998). The objective of this study was to evaluate the effects of cotinine or CS on the formation of promutagenic O°MeG adducts in A/J mice during the acute phase of NNK treatment.

MATERIALS AND METHODS

Chemicals

NNK was purchased from Chemsyn Science Laboratories (Lenexa, KS). Guanine (Gua), O°MeG and cotinine were purchased from Sigma Co. (St. Louis, MO); the latter was redistilled to reach a purity greater than 99% as determined by gas chromatography. 1R4F reference cigarettes were purchased from the Tobacco and Health Institute of the University of Kentucky (Lexington, KY) and were stored at 4°C until use. The cigarettes were unpacked and conditioned for 24–72 h in a Climate-Lab® cabinet (PGC, Black Mountain, NC) at a 40–70% relative humidity and a temperature of 18–26°C prior to smoking.

Inhalation Exposure System

Smoke generator. Mainstream smoke from 1R4F reference cigarettes was generated using a 30-port, computer-controlled smoke generator (Ayres *et al.*, 1990) with automatic load and ejection capabilities. Mainstream smoke was generated using the Federal Trade Commission (FTC) puffing regimen of a 2-s puff with 35 ml volume, and a frequency of one puff per min. Puff volume was measured with a soap bubble flow meter before the inhalation exposure, and a peristaltic pump was used to create the vacuum for the puff. Smoke from the peristaltic pump was directed into a mixing flask where HEPA-filtered air (through a Del-Monox Compressed Air Purification system [Deltech Engineering, New Castle, DE]), at a flow rate of approximately 600 ml/min, was mixed with the cigarette smoke. Diluted smoke then entered another flask where approximately 25 L/min of HEPA-filtered and humidified air entered the mixing vessel. Controlled concentrations of diluted smoke (at 0.4, 0.6, or 0.8 mg of wet total particular matter/liter of air [WTPM/L]) were then directed to the nose-only exposure chamber. The exposure atmosphere had a relative humidity of 40–70% and a temperature of 18–26°C.

Nose-only exposure chamber. The nose-only exposure chamber consisted of three circular tiers with 24 exposure ports per tier. The flow rate passing through the nose-only exposure chamber was adjusted to 25 L/min to provide approximately 300 ml/min/port of air at each exposure port. Before smoke exposures were initiated, performance of the nose-only smoke exposure was evaluated to confirm that the coefficients of variance for the average air flow rate within a tier, flow rate among tiers, temporal stability and between-port variability were 3%, 2%, 1%, and 2%, respectively.

Exposure concentration and duration. The concentration of smoke in the nose-only exposure chamber was controlled by a feedback-loop exposure controller. Breathing zone smoke concentrations were monitored by a RAM-1 aerosol concentration analyzer (MIE Corp., Billerica, MA). The analog signal from a RAM-1 was directed to a computer control unit. Analog voltage, indicative of the smoke concentration in the exposure chamber, was compared to the target voltage, which in turn was related to the desired exposure concentration. If the analog voltage from the RAM-1 was not in agreement with the target voltage, a 2 LPM mass flow controller operating in the vacuum mode (attached at the tee as previously described) altered the amount of smoke from the peristaltic smoke pump available for final dilution and delivery to the chamber.

Physical characterization of smoke aerosol. The particle size distribution was monitored by time of flight analysis using an aerodynamic particle sizer (APS) (model APS33B, TSI, St. Paul, MN). For our experiments, the APS had 48 effective cutoff diameters (ECD) in the particle size range of 0.5–14.9 μm and 22 ECD in the size range of 0.5–2.28 μm. Calculations of mass median aerodynamic diameter and geometric standard deviation were based on a log-normal distribution.

The exposure concentrations used in this study were 0, 0.4, 0.6, or 0.8 mg WTPM/L. These concentrations were achieved and equilibrated in a bypass exposure system before exposure of mice. During equilibration of the smoke concentration, mice in nose-only restraint tubes were placed on the nose-only exposure system and supplied HEPA-filtered and humidified breathing air. Once a stable concentration was verified, the smoke was directed to the nose-only exposure system.

Animals and Treatment

Six to seven-week-old female A/J mice, purchased from Jackson Laboratory (Bar Harbor, ME), were housed singly and acclimated in an environment providing a 12-h light/dark cycle. The room temperature and humidity were 20–25°C and 55 ± 15%, respectively. Mice had *ad libitum* access to water and

pelleted AIN-76A diet (Dyets, Inc., Bethlehem, PA). After acclimation for 14 days, animals were allocated into groups with no statistical differences in group-mean body weights, and the experiment was started.

Effect of cotinine on NNK-induced adducts. To avoid cotinine toxicity, cotinine was administered at 50 μmole/mouse in 3 separate doses: 30 min before (25 μmole/mouse), immediately after (12.5 μmole/mouse), and 30 min after (12.5 μmole/mouse) a single intraperitoneal dose of NNK (0, 2.5, 5, 7.5, or 10 μmole/mouse). Control animals received physiological saline instead of cotinine. Four hours after NNK treatment, mice were sacrificed by inhalation of 70% CO_2 in air. Lung and liver tissues were collected in sample vials, quick-frozen in liquid nitrogen, and stored at −70°C until DNA isolation.

Effect of cigarette smoke on NNK-induced adducts. The initial study varied the smoke concentration and kept the NNK dose constant. Mice received nose-only inhalation of CS at 0, 0.4, 0.6, or 0.8 mg WTPM/L for 2 h and NNK (7.5 μmole/mouse, ip) was administered at the midpoint of the 2-h exposure. NNK exposure from smoke inhalation ranged from 0 to approximately 18 ng per mouse. Mice were euthanized 4 h after NNK treatment.

In a subsequent study, mice received a one-time, nose-only inhalation regimen of either HEPA-filtered and humidified air (control) or 1R4F CS at a concentration of 0.6 mg WTPM/L for 2 h to monitor the effect of CS on the concentration of O⁶MeG in mice treated with NNK. A single ip dose of NNK (0, 3.75, or 7.5 μmole/mouse) was administered to mice at the midpoint of the 2-h exposure. NNK exposure from 0.6 mg WTPM/L for 2 h was approximately 13.5 ng per mouse. Mice were euthanized 4 h after the NNK treatment.

Euthanization and sample collection. Surviving mice were euthanized by inhalation of 70% CO_2 in air. Lung and liver tissues were collected in sample vials, quick-frozen in liquid nitrogen and stored at −70°C until DNA isolation.

Sample Analysis

Sample preparation. Lung and liver were thawed on ice, weighed and homogenized in 2 ml of ice-chilled Hank's balanced salt solution (HBSS). The DNA was isolated using the Genpure™ 341 Nucleic Acid Purification System (Applied Biosystems, Foster City, CA) and the purified DNA pellet was stored at −70°C until hydrolyzed. Just prior to hydrolysis, the DNA pellet was dissolved in 230 μl of deionized water by incubating the resuspended DNA in an 80°C water bath for 20 min. At the end of the 20-min incubation, 5 μl of dissolved DNA was diluted with deionized water (495 μl), checked for purity at 260 and 280 nm, and quantified by spectrophotometry at 260 nm. Following determination of the DNA concentration, the remaining DNA solution (0.5–2 mg) was hydrolyzed in 0.1 N HCl for 30 min at 80°C. The hydrolysate was chilled on ice and centrifuged at 10,000 × g for 5 min to remove the pelleted particulates. Hydrolyzed DNA samples were analyzed by HPLC for methylated bases within 16 h of hydrolysis.

Quantification of methylated DNA bases with HPLC. Quantification was conducted as described below using a modification of previous methods (Herron and Shank, 1979; Bedell *et al.*, 1982; Hecht *et al.*, 1986b). Typical chromatograms using this modified method are illustrated in Figure 2. For sample analysis, approximately 0.25–1 mg of acid-hydrolyzed DNA sample was injected (Waters 717 Plus Autosampler) into the mobile phase (10% methanol and 90% 50 mM $NH_4H_2PO_4$, pH 2.0), which was pumped at a flow rate of 1.5 ml/min by a Waters 626 pump assisted by a Waters 600S Controller (Waters Co., Milford, MA). Separation was carried out by two in-line Partisil® 10-SCX columns (4.6 × 250 mm, 5 μm, Whatman, Inc., Clifton, NJ) placed in series. Guanine was detected by a Waters 996 Photodiode Array Detector (absorbance set at 226 nm) and O⁶MeG was detected by a Waters 470 Scanning Fluorescence Detector (using a 16-μl flow-through cuvette; excitation λ:286 nm and emision λ:366 nm; gain at 1000×; filter at 1.5 nm; window slit at 18 nm). All signals were integrated using Waters Millennium® Software (Waters Co., Milford, MA). Peak responses were then quantified using calibration curves generated from freshly-prepared Gua and O⁶MeG standards dissolved in 0.1 N HCl. Signals below the limit of detection were interpreted as "0" for statistical analysis.

Authentic standards of guanine and O⁶MeG were prepared fresh in 0.1 N HCl for each batch of HPLC analyses. The mixture was injected 4 times at the beginning of the run and 4 times at the end of the run. The ratios of guanine/O⁶MeG were quantified and variations within each run and among those runs were monitored for reproducibility. Standards were injected and integrated by HPLC for calibration range finding. HPLC precision was determined within each batch run (intra-assay precision) and among batches (inter-assay) throughout the studies.

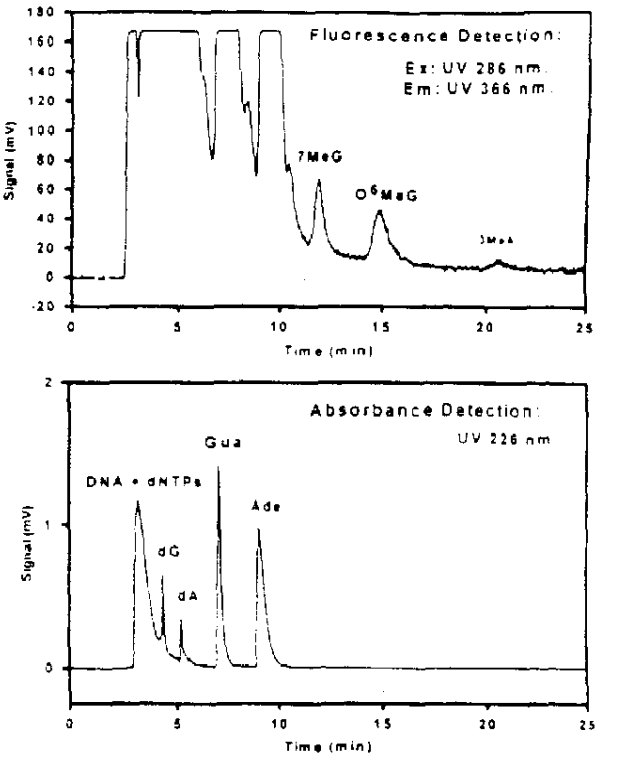

FIG. 2. HPLC chromatograms of thermal/acid-hydrolyzed liver DNA from an NNK-treated mouse. Thermal/acid-hydrolzyed DNA sample was quantified using HPLC equipped with two in-line, cation-exchange-based analytical columns. Guanine, the normalizing molecule for quantifying O⁶MeG, was detected by an absorbance detector (lower); O⁶MeG was detected by a fluorescence detector (upper).

Statistics

Two-sample Student's *t*-test was applied to assess the difference between groups, and the significance level was set at $p < 0.05$. A dose-response analysis was performed, comparing the slopes of the NNK effects for mice exposed to cigarette smoke versus mice exposed to filtered air. The dose responses were calculated as the difference of the two orthogonal linear contrasts for the NNK effect. For animals given a fixed amount of NNK, then exposed to varying amounts of cigarette smoke, a comparison was made at each dose level of cigarette smoke to the filtered air-exposed animals with Bonferroni (Krewski and Franklin, 1991) adjustments made for multiple comparisons. For animals that were given varying doses of NNK, a comparison was made between mice exposed to cigarette smoke and mice exposed to filtered air at each NNK dose level (making a Bonferroni adjustment for multiple comparisons). Results were examined to determine if there was a

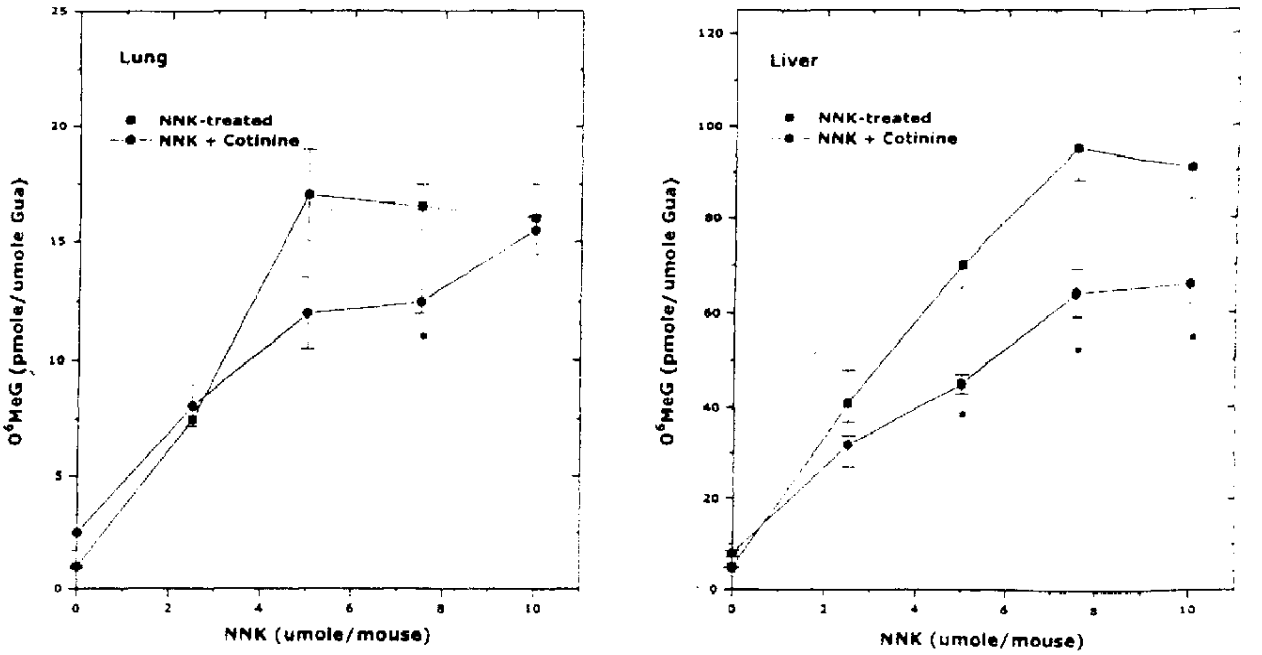

FIG. 3. Effect of cotinine (50 μmole/mouse) on the lung and liver concentrations of O⁶MeG in NNK-treated A/J mice. Cotinine was administered in 3 separate injections, 30 min before [25 μmole/mouse], immediately after [12.5 μmole/mouse], and 30 min after [12.5 μmole/mouse] NNK treatment at 5, 7.5, and 10 μmole/mouse. Mice were euthanized 4 h after NNK treatment and O⁶MeG was quantified in lung and liver by HPLC. (Mean ± SE; $n = 18$; * = $p < 0.05$.)

dose-response for cigarette smoke, which was calculated from the orthogonal linear contrast involving the cigarette smoke exposures.

The variance was different among the different treatment groups; accordingly, a Satterthwaite approximation was used to determine degrees of freedom for each of the comparisons made.

RESULTS

The instrument calibration range for guanine and O⁶MeG was 0.01–300 nmole and 0.4–100 pmole, respectively (data not shown). The HPLC precision for the quantification of O⁶MeG was 1.16% (CV) for intra-assays and 4.54% (CV) for inter-assays.

The cotinine treatment regimen (a total of 50 μmole/mouse given in 3 separate injections, 30 min before, immediately after, and 30 min after NNK treatment at 5, 7.5 or 10 μmole/mouse) reduced liver concentrations of NNK-induced O⁶MeG up to 35% four hours after NNK injection (Fig. 3). In the lung, we observed a significant reduction of O⁶MeG at a NNK dose of 7.5 μmole/mouse ($p < 0.05$) and a trend of reduction is present at the NNK dose of 5.0 μmole/mouse, though not statistically significant. Interestingly, at the 10 μmole/mouse NNK dose, cotinine showed no effect on the NNK-induced O⁶MeG concentration (Fig. 3).

A/J mice were exposed to cigarette smoke concentrations ranging from 0.4 to 0.8 mg WTPM/L in a 2-h, nose-only regimen. Our studies demonstrate that cigarette smoke exposure alone failed to induce detectable levels of O⁶MeG in A/J mouse lung and liver, consistent with a previous study by Finch *et al.* (1996) demonstrating that no lung tumors were induced in A/J mice by cigarette smoke. Consistent with previous studies (Peterson and Hecht, 1991), a single ip injection of NNK (7.5 μmole/mouse) produced O⁶MeG DNA adducts in both lung and liver (Figs. 3 and 4). However, exposure to cigarette smoke reduced the concentration of O⁶MeG in lung and liver in a dose-dependent fashion (Fig. 4). Due to the high mortality during exposure of mice to 0.8 mg WTPM/L (Table 1), the exposure level of 0.6 mg WTPM/L, which caused minimal mortality, was recognized as the MTD for the A/J mice in a subsequent 2-h, nose-only 1R4F cigarette smoke inhalation exposure regimen to examine the effect on the NNK response. In this study, O⁶MeG adduct formation in both lung and liver was dependent on the dose of NNK and cigarette smoke was effective in reducing adduct formation in both tissues with a maximal reduction of 46% in lung and 52% in liver (Fig. 5).

DISCUSSION

Cigarette smoke is a complex mixture consisting of more than 3,500 chemicals including NNK, a tobacco-specific nitro-

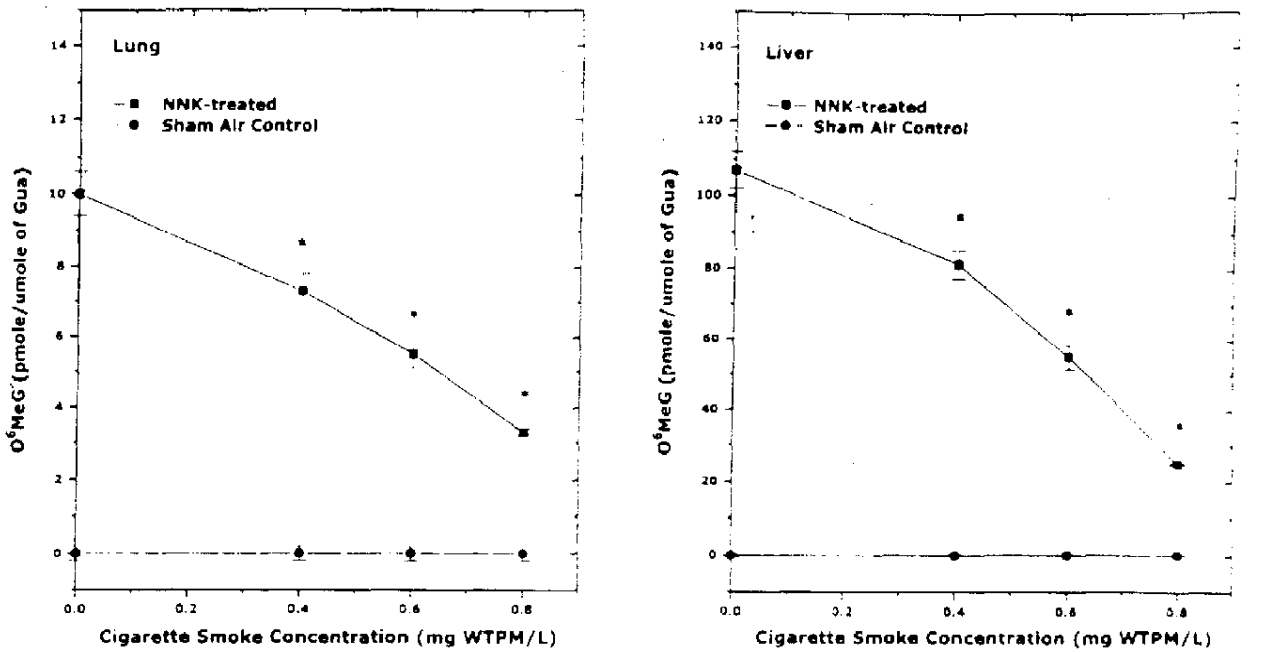

FIG. 4. Dose-dependent reduction of the lung O⁶MeG concentration by 1R4F cigarette smoke in A/J mice. Mice received a one-time, nose-only inhalation exposure of 1R4F cigarette smoke at 0, 0.4, 0.6, or 0.8 mg WTPM/L for 2 h to study the potential of CS to inhibit NNK-induced O⁶MeG formation. The dosing of NNK (7.5 μmole/mouse, ip) was performed at the midpoint of the 2-h exposure. Mice were euthanized 4 h after NNK treatment and lung and liver DNA was analyzed for O⁶MeG by HPLC. (Mean ± SE; $n = 18$; * = $p < 0.05$.)

samine. Studies examining the potential role of NNK in cigarette smoke tumorigenicity are frequently carried out by dosing animals with this nitrosamine to demonstrate tumorigenicity in target organs (Hecht *et al.*, 1989). These studies are generally conducted by injecting rodents with 5 to 10 μmoles of NNK. The amount of NNK in a Kentucky reference 1R4F cigarette, which is designed to represent the U.S. market, is 67 ng/cigarette (Borgerding *et al.*, 1997). Therefore, the amount of NNK used to demonstrate carcinogenicity in A/J mice is equivalent to the amount of NNK extracted from the mainstream smoke of 15,000 to 30,000 cigarettes. Although highly exaggerated doses are often used in rodent carcinogenicity studies, the NNK carcinogenicity studies could be criticized in that the experimental design employed fails to account for the inhibitory or stimulatory effect(s) of other chemicals in the tobacco smoke mixture on the carcinogenicity of NNK. Hence, it is more appropriate that the role of NNK in the toxicity and/or potential carcinogenicity of tobacco smoke be evaluated in a test system concurrently dosed with cigarette smoke, or at least some of its components.

TABLE 1
Mortality of NNK-Treated A/J Mice Receiving 1R4F Cigarette Smoke

NNK dose	Sham air	0.4 mg WTPM/L	0.6 mg WTPM/L	0.8 mg WTPM/L
0	0%	0%	0%	11%
3.75 μmole/mouse	0%	0%	0%	17.8%
7.5 μmole/mouse	0%	0%	5%	52%

Note. Mice were exposed nose-only to cigarette smoke for 2 h. NNK was administered to the mice at the midpoint of the 2-h exposure. Mortality of the mice was determined 4 h after NNK treatment ($n = 18$).

Metabolism of nicotine to cotinine *in vivo* occurs within minutes after nicotine absorption, with cotinine having a half-life of 19–24 h in rodents (Rama Sastry *et al.*, 1995). Hence, nicotine absorbed internally from cigarette smoke is rapidly converted to cotinine, which then resides within the body for a much longer period than nicotine. The current study demonstrates that cotinine significantly reduces the number of O⁶MeG DNA adducts produced by injected NNK. Previous studies have shown that α-hydroxylation of NNK is catalyzed mainly by cytochromes P_{450} (Hecht, 1996). In mouse lung, CYP2A1 and CYP2B1 appear to be the cytochromes P_{450} that act as catalysis of α-hydroxylation. In the mouse liver, CYP1A2, CYP2A6, and CYP2E1 play prominent roles (Hecht, 1996). We hypothesize that the reduction of O⁶MeG in liver and lung is due to competitive inhibition by cotinine of the cytochrome P_{450} enzyme system for NNK activation. In ciga-

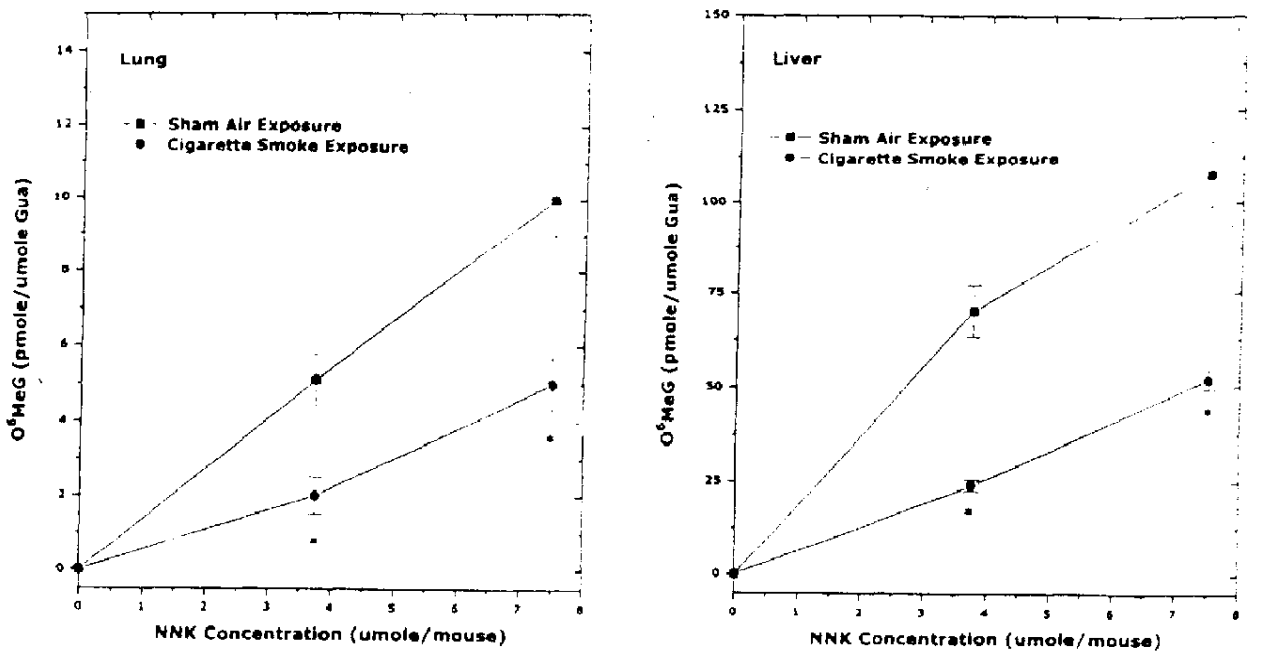

FIG. 5. Effect of 1R4F cigarette smoke (0.6 mg WTPM/L) on the lung and liver concentrations of O⁶MeG in NNK-treated A/J mice. Mice received a one-time, nose-only inhalation regimen of either HEPA-filtered and humidified air (control) or 1R4F cigarette smoke at the previously determined MNLD (0.6 mg WTPM/L) for 2 h to monitor the effect of cigarette smoke on the concentration of O⁶MeG in mice treated with NNK. A single ip dose of NNK (0, 3.75, or 7.5 μmole/mouse) was administered to mice at the midpoint of the 2-h exposure. Mice were euthanized 4 h after the NNK treatment, lung and liver DNA was analyzed for O⁶MeG by HPLC. (Mean ± SE; $n = 18$; * = $p < 0.05$.)

rette smoke, the ratio of nicotine to NNK has been reported to vary between 3,000:1 and 30,000:1 (Richter and Tricker, 1994). In the present study, the mass ratio between cotinine and NNK is limited, due to a necessity to deliver a sufficiently large dose of NNK to generate amounts of O⁶MeG detectable through HPLC, while at the same time keeping the cotinine below toxic levels. Our results indicate that cotinine, at a mass ratio to NNK as low as 5:1, significantly ($p < 0.05$) reduces the concentration of O⁶MeG produced by NNK in A/J mouse liver, and ratios as low as 6.67:1 significantly ($p < 0.05$) reduces the concentration of O⁶MeG produced by NNK in A/J mouse lung. This reduction might be much greater within the situation of real smoke, given that the cotinine/NNK ratio in cigarette smoke is approximately 500 times higher than the ratios we used in this study. This hypothesis is supported by our observation that O⁶MeG adducts were not detected in mice acutely exposed to a maximum tolerated dose (MTD) of cigarette smoke. This data is consistent with tumorigenesis results previously reported by Finch *et al.* (1996). Those studies demonstrated that no lung tumors were induced or promoted in A/J mice by cigarette smoke at a concentration of 248 mg total particulate matter/m^3 for 6 h/day, 5 days/week, for 26 weeks. In our studies, cigarette smoke exposure concurrent with ip NNK administration significantly reduced the formation of O⁶MeG in a dose-dependent fashion, with a maximum reduction of 46% and 52% in lung and liver, respectively. These results are consistent with the results of Hecht *et al.* (1983), which indicated that exposure to cigarette smoke increased the survival rate in NNK-treated rodents compared to controls exposed to air.

This study demonstrates for the first time that cotinine, the major metabolite of nicotine, as well as cigarette smoke effectively reduce the level of promutagenic DNA adducts induced by NNK. This study provides a mechanistic basis for previous dichotomous observations that NNK, but not tobacco smoke, induces lung adenomas in the A/J mouse.

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NOTE ADDED IN PROOF

A recent article (*Experimental Lung Research* **24,** 385–394, 1998) reported that exposure to tobacco smoke can, under certain experimental conditions, reproducibly increase the lung tumor multiplicity in A/J mice, although there was not a reproducible increase in lung tumor incidence. Consistent with the results reported here, the authors conclude that NNK is not likely to be a causative agent for the observed increase in tumor multiplicity.